Light-induced synthesis of protein conjugates and its application in photoradiosynthesis of 89Zr-radiolabeled monoclonal antibodies

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Abstract: Efficient methods to functionalize proteins are essential for the development of many diagnostic and therapeutic compounds, such as fluorescent probes for immunohistochemistry, zirconium-89 radiolabeled mAbs (89Zr-mAbs) for positron emission tomography and antibody-drug conjugates (ADCs). This protocol describes a step-by-step procedure for the light-induced functionalization of proteins with compounds bearing the photochemically active aryl azide group. As an illustration of the potential utility of our approach, this protocol focuses on the synthesis of 89Zr-mAbs using photoactivatable derivatives of the metal ion binding chelate desferrioxamine B (DFO). The light-induced synthesis of 89Zr-mAbs is a unique, one-pot process involving simultaneous radiolabeling and protein conjugation. The photoradiochemical synthesis of purified 89Zr-mAbs, starting from unmodified proteins, [89Zr][Zr(C2O4)4]4– (89Zr-oxalate), and a photoactivatable DFO derivative, can be performed in <90 min. The method can be easily adapted to prepare other radiolabeled proteins, ADCs or fluorescently tagged proteins by using drug molecules or fluorophores functionalized with photoactive moieties.

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Light-induced synthesis of protein conjugates and its application in photoradiosynthesis of ^{89}Zr-radiolabelled monoclonal antibodies

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KEYWORDS Protein conjugation, photochemistry, radiochemistry, photoradiosynthesis, positron emission tomography (PET), antibodies, zirconium-89.

EDITORIAL SUMMARY This protocol describes the light-induced functionalisation of proteins with compounds bearing the photochemically active aryl azide (ArN_3) group. The procedure is exemplified by the light-induced radiosynthesis of ^{89}Zr-monoclonal antibodies.

TWEET A new Protocol for light-induced protein functionalisation and its application in radiosynthesis of ^{89}Zr-monoclonal antibodies @UZH_Chemistry, @UZH_Science

COVER TEASER Light-induced protein functionalisation in a flash

ABSTRACT

Efficient methods to functionalise proteins are essential for the development of many diagnostic and therapeutic compounds such as fluorescent probes for immunohistochemistry, zirconium-89 radiolabelled monoclonal antibodies (^{89}Zr-mAbs) for positron emission tomography (PET), and antibody-drug conjugates (ADCs). This protocol describes a step-by-step procedure for the light-induced functionalisation of proteins with compounds bearing the photochemically active aryl azide (ArN_3) group. As an illustration of the potential utility of our approach, this protocol focuses on the synthesis of ^{89}Zr-mAbs using photoactivatable derivatives of the metal ion binding chelate desferrioxamine B (DFO). The light-induced synthesis of ^{89}Zr-mAbs is a unique, one-pot process involving simultaneous radiolabelling and protein conjugation. The photoradiochemical synthesis of purified ^{89}Zr-mAbs, starting from unmodified proteins, $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ (^{89}Zr-oxalate), and a photoactivatable DFO derivative, can be performed in less than 90 minutes. The method can be easily adapted to prepare other radiolabelled proteins, ADCs or fluorescently-tagged proteins using drug molecules or fluorophores functionalised with photoactive moieties.

INTRODUCTION

Protein-based drugs and related biological products (biologics) represent one of the fastest growing sectors in the pharmaceutical industry. The versatile structure and function of proteins make them ideal scaffolds for designing sophisticated drugs that exhibit high affinity, target specificity, and optimised pharmacokinetics. Monoclonal antibodies (mAbs) and immunoglobulin fragments are frequently used as protein components in many biologics designed as therapeutic or diagnostic agents. For example, mAbs functionalised with fluorophores are used as biochemical tools in immunohistochemistry, fluorescence-assisted cell sorting or confocal fluorescence microscopy; antibody-drug conjugates (ADCs) are among the latest generation of targeted chemotherapeutics; and ^{89}Zr ($t_{1/2}$ = 78.41 h) radiolabelled mAbs are a cornerstone of diagnostic PET imaging in Nuclear Medicine.

The synthesis of radiolabelled mAbs (and other protein-drug conjugates) requires chemical modification of the glycoprotein.[1] Standard protein coupling chemistries rely almost exclusively on thermochemically induced reactions such as the post-translational modification of amino-acid side chains or the site-specific labelling of glycans by chemical or enzymatic methods.[2–10] Although highly successful, these classic approaches suffer from a number of important limitations. For instance, most existing methods are time consuming and difficult to automate. Chemical reagents used for conjugation to amine (-NH_2), carboxylate (-CO_2^-), or sulfhydryl (-SH) groups frequently involve multiple step syntheses and are incompatible with protein formulation buffers which contain various redox stabilisers, amino acids, sugars and other solubilising agents. As an example, an outstanding *Nature Protocol* by Vosjan *et al*.[11] describes the two-step protein conjugation and radiosynthesis of ^{89}Zr-mAbs using *p*-isothiocyanatobenzyl-desferrioxamine B (DFO-Bn-NCS).[12] A covalent, thiourea linkage is formed between the NCS group of the chelate

and available primary amines on the protein (mainly the ε-NH_2 side-chain of lysine) at pH 9. DFO-Bn-NCS conjugation to mAbs is highly reproducible with conjugation efficiencies of 60% to 80%. However, the reaction conditions do not tolerate the presence of free amino acids. Hence, pre-purification of the mAb from other formulation components is necessary for functionalisation using Bn-NCS chemistry. Standard thermochemical routes also require the isolation, characterisation, and storage of an intermediate new molecular entity (the conjugated mAb), which poses both practical and economic problems during clinical translation.

Development and Overview of the Protocol

An ideal method for protein-conjugation should be a simple, fast and reproducible process that can be performed in a single step under mild conditions using well-characterised, stable precursors. In 2019, our group demonstrated that light-induced activation of chelates bearing aryl azide (ArN_3) groups and radiolabelled with a variety of different radionuclides, including $^{68}Ga^{3+}$ and $^{89}Zr^{4+}$ ions, can produce viable PET radiotracers direct from formulated antibody solutions.[13–20] The underlying concept of *bimolecular* photochemically induced protein modification is shown in **Figure 1**.[19,21]

The key motivation for developing an alternative protein conjugation and radiolabelling strategy was to eliminate steps that involved pre-purification of the protein and isolation of a functionalised (non-radiolabelled) intermediate molecule. The functionalised intermediates that are produced from existing methodologies can be categorised as New Molecular Entities (NMEs) and in some jurisdictions are subject to stringent absorption, distribution, metabolism excretion and toxicological (ADME-tox) testing. Aside from the very high economic costs, obtaining sufficient amounts of material to perform these ADME-tox studies is a major technological and

logistical challenge that prohibits many smaller research groups and facilities from developing ^{89}Zr-mAbs.

By minimising the number of steps, reducing reaction times, and by performing the bioconjugation chemistry in the presence of standard formulation buffers, we were able to reduce the risk of compromising the integrity of the sensitive biological materials (antibodies and other proteins). Photochemical methods provide access to a unique set of covalent linkages (here the ArN_3 produces a heterocyclic azepin group) which offers new opportunities for designing protein-conjugates with advanced functionality for controlled drug release.

Advantages and Limitations

Our recent experiments have shown that a photoradiosynthetic approach to produce ^{89}Zr-mAbs from ArN_3-based reagents has several advantages over existing methods. These include: *i*) rapid reaction kinetics – the protein conjugation and radiolabelling steps occur simultaneously, under the same conditions, and the entire one-pot reaction is performed in <15 min; *ii*) the photochemical conjugation process is tolerant to water, oxygen, salts and clinical-grade mAb formulation buffers that can include high concentrations of free amino acids (such as histidine), sugars and surfactants (such as polysorbate 20); *iii*) photoactivation occurs at wavelengths (365 – 395 nm) where antibodies do not absorb; and *iv*) ^{89}Zr-mAbs can be synthesised directly from formulated stocks avoiding pre-purification of the protein and isolation of intermediates; others have made important progress on automated methods for producing ^{89}Zr-mAbs,[22] yet the chemistry still requires the use of pre-functionalised intermediates. In contrast, the photoradiochemical approach is amenable to full automation.

In spite of the many attractive features, photochemical synthesis of protein-conjugates involves several limitations that are distinct from standard thermochemical processes. The light-induced chemistry is highly dependent on the experimental geometry. Kinetic studies have shown that for compounds bearing an ArN_3 group, the photo-initiation step is highly efficient and linearly dependent on photon flux. However, quantum yields for photochemical activation depend on the photon beam shape, focal point, and reaction volume. Scattering or absorption of the incident light by the reaction vessel or chemical components in the mixture can also have a dramatic impact on the observed conjugation yields. Experimentally, radiochemical yields (RCYs) of ^{68}Ga- or ^{89}Zr-mAbs have also shown a steep dependence on pH and the initial protein concentration.[13,15,18,20] This protocol introduces the procedure for performing simultaneous, one-pot photoradiolabelling of ^{89}Zr-mAbs and some of the considerations that must be taken into account when performing photochemistry in the context of protein conjugation.

Applications

The primary application envisaged for this protocol is the synthesis of radiolabelled proteins, and in particular ^{89}Zr-mAbs, for use in diagnostic and nuclear medicine procedures. In recent work, we demonstrated that photoradiochemistry can also be used with different radioactive metal ions (published: ^{68}Ga, ^{89}Zr, ^{111}In; unpublished: ^{64}Cu, ^{99m}Tc, ^{177}Lu), metal binding chelates (DFO, NOTA, NODAGA, DOTA, DOTAGA, DTPA, HBED-CC), and proteins including serum proteins like human serum albumin (HSA), immunoglobulins (IgG_1: trastuzumab; Herceptin™), and engineering protein constructs (scFv-Fc fragments including onartuzumab; MetMAb™).[13–19] Radiochemical yields varied substantially depending on the reaction parameters.

We note that the chemistry is not limited to the production of radioactive materials. Our recent work on the development of photoactivatable fluorophores (PhotoTags) demonstrated that protein functionalisation with BODIPY-ArN_3 can be achieved by using a similar protocol.[21] In addition, we are actively working on the synthesis of ADCs using photochemical methods to link cytotoxic payloads to a variety of biochemical vectors.

Experimental Design

As an example, this protocol describes the one-pot photoradiosynthesis of [^{89}Zr]ZrDFO-PEG_3-azepin-onartuzumab.[20] Prior to commencing work, it is important have access to a suitable photoactivatable chelate. Details of the chemical synthesis and characterisation of DFO-PEG_3-ArN_3 (**1**) are provided in **Box 1**. Further details on the synthesis and characterisation of other photoactivable chelates, including compounds **2** and **3**, are reported elsewhere (Figure 2).[13–20] We note that during the development of this protocol, variations in radiochemical yields (RCY) were obtained. The most important factors that influence the final RCY include the nature of the protein undergoing functionalisation, the composition of the formulation, and particularly the protein concentration in the reaction mixture.[18] Optimisation is likely required for each independent protein to obtain the maximum protein conjugation efficiency and highest RCYs. Furthermore, the experimental geometry has a major influence on the success and reproducibility of the procedure. Prior to using the protocol to synthesise ^{89}Zr-mAbs, optimisation of the experimental set-up should be performed to ensure maximum delivery of the light into the reaction mixture. Dedicated photochemical reactors exist but to facilitate the use of the protocol, the chemistry was performed by using a simple experimental design that is accessible to standard chemistry and radiochemistry facilities (Figure 3).

BOX 1 – Synthesis and Characterisation of DFO-PEG$_3$-ArN$_3$ (1) • TIMING 6 days

This box describes the synthesis of photoactivatable DFO-PEG$_3$-ArN$_3$ **(1)** according to the schematic shown below (adapted from Ref. [20]).

[PE: Please place Box 1 Figure 1 here]

REAGENTS

CRITICAL: All reagents purchased from commercial sources were used without further purification.

- 4,7,10-Trioxa-1,13-tridecanediamine (CAS no. 4246-51-9; Merck, cat. no. 369519)…
- di-*tert*-butyl dicarbonate (Boc_2O, CAS no. 24424-99-5; Merck, cat. no. 361941)
- 4-azidobenzoic acid (CAS: 6427-66-3, Merck, cat. no. 778877)
- 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, (HATU, CAS: 148893-10-1, Merck cat. no. 11373)
- *N,N*-diisopropylethylamine (DIPEA, CAS: 7087-68-5, Merck, cat. no. D125806)
- $MgSO_4$ (CAS: 7487-88-9, Merck, cat. no. M7506)
- Trifluoroacetic acid (CAS: 76-05-1, Merck, cat. no. T6508)
- $NaHCO_3$ (CAS: 144-55-8, Merck, cat. no. S8875)
- Succinic anhydride (CAS: 108-30-5, Merck, cat. no. 239690).
- DFO mesylate (CAS: 138-14-7, Merck, cat. no. D9533)

PROCEDURE

CRITICAL The syntheses of intermediate compounds **4**, **6**, **7** and **8** are adapted from Patra *et al.*, Angew. Chemie Int. Ed., **58**, 1928–1933 (2019). DOI: 10.1002/anie.201813287.[13]

1. Synthesize compound **4** (*tert*-butyl 3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propylcarbamate) by following the procedure reported by Liu *et al*.[23] Briefly, a solution of 4,7,10-Trioxa-1,13-tridecanediamine in CH_2Cl_2 and di-*tert*-butyl dicarbonate in CH_2Cl_2 was reacted overnight under Ar(g). The mixture was concentrated under reduced pressure, washed with sat. $NaHCO_3$, and dried over $MgSO_4$.
2. Synthesize compound **6** (*tert*-butyl (1-(4-azidophenyl)-1-oxo-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate) by reacting a stirred solution of 4-azidobenzoic acid (compound **5,** 1.0 g, 6.13 mmol) and 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate, (HATU, 3.5 g, 9.2 mmol) in dry dimethylformamide (DMF, 8 mL) with *N,N*-diisopropylethylamine (DIPEA, 3.16 g, 24.6 mmol). After 40 minutes stirring at 23 ^{o}C under N_2 atmosphere, add compound **4** (3.93 g, 12.3 mmol) dropwise in DMF (4 mL) and stir the mixture for 24 h. Monitor the reaction by TLC (silica gel, EtOAc). Remove the solvent under reduced pressure and dissolve the residue in CH_2Cl_2 (150 mL). Wash the organic layer with H_2O (3 × 100 mL) and brine (2 × 100 mL). Dry the residue over $MgSO_4$, followed by filtration and removal of the solvent under reduced pressure. Purify the crude sample by column chromatography (silica, EtOAc) to give compound **6** as a slightly yellow oil (2.43 g, 88%). Characterisation data including ^{1}H- and ^{13}C{^{1}H}-NMR spectroscopy, high-resolution electrospray ionisation mass spectrometry (HRMS) and reverse-phase high-performance liquid chromatography (HPLC) are reported elsewhere.[13,20]

3. Deprotect compound **6** by dissolving compound **6** (1.30 g, 2.79 mmol) in CH_2Cl_2 (5 mL), add trifluoroacetic acid (3 mL) at 0 °C, and then stir the mixture overnight. Wash the organic layer with sat. $NaHCO_3$(aq) (3 × 30 mL; powdered form of $NaHCO_3$(s), dry over $MgSO_4$ and remove the solvent under reduced pressure to give compound **7** as a yellow oil (1.01 g, 99%). Characterisation data including ^{1}H- and ^{13}C{^{1}H}-NMR spectroscopy, high-resolution electrospray ionisation mass spectrometry (HRMS) and reverse-phase high-performance liquid chromatography (HPLC) are reported elsewhere.[13,20]
4. Synthesize compound **8** by reacting a stirred solution of compound **7** (348 mg, 0.952 mmol) in DMF (5 mL) with succinic anhydride (143 mg, 1.43 mmol). Stir the mixture for 48 h at room temperature under an atmosphere of N_2. After evaporation of the solvent, purify the crude product by flash chromatography (C18, H_2O to H_2O/MeOH 5/95) to give compound **8** as a yellow oil (290 mg, 66%). Characterisation data including ^{1}H- and ^{13}C{^{1}H}-NMR spectroscopy, high-resolution electrospray ionisation mass spectrometry (HRMS) and reverse-phase high-performance liquid chromatography (HPLC) are reported elsewhere.[13,20]
5. Synthesize the photoactivatable compound DFO-PEG_3-ArN_3 (compound **1**) by standard amide bond formation: First, dissolve compound **8** (690 mg, 1.48 mmol) and HATU (844 mg, 2.22 mmol) in DMF (100 mL). Then, add DIPEA (1.03 mL, 5.92 mmol) and stir the mixture for 45 min at room temperature, followed by the addition of DFO mesylate (1.17 g, 1.78 mmol). Stir the mixture for 48 h before evaporation of the solvent under reduced pressure. Purify the crude product by flash chromatography (C18, 135 g, H_2O to H_2O/MeOH 5/95). Combine fractions containing the desired compound and remove the

solvent under reduced pressure. Obtain a purified sample of the product by dialysis and lyophilisation to give compound **1** as a pale-yellow amorphous powder (1.02 g, 69%).

CRITICAL STEP: Analytical Data for compound **1**: **^{1}H NMR** (DMSO-d^{6}, 400 MHz, 298 K) δ (ppm) 1.04-1.34 (m, 6H, CH_2), 1.37-1.40 (m, 4H, CH_2), 1.48-1.51 (m, 4H, CH_2), 1.57-1.62 (m, 2H, CH_2), 3.0-3.12 (m, 6H, CH_2), 3.30-3.57 (m, 20H, CH_2), 7.19 (d, 2H, ^{3}J = 8 Hz, CH_{Ar}), 7.76 (br s, 3H, N-O*H*), 7.88 (d, 2H, ^{3}J = 8 Hz, CH_{Ar}), 8.44 (br s, 1H, N*H*), 9.66 (br s, 2H, N*H*). **^{13}C{^{1}H} NMR** (DMSO-d^{6}, 101 MHz, 298 K) δ (ppm) 20.78 (CH_3), 23.9 (CH_2), 26.5 (CH_2), 28.0 (CH_2), 29.3 (CH_2), 29.8 (CH_2), 30.4 (CH_2), 31.3 (CH_2), 31.4 (CH_2), 36.3 (CH_2), 37.2 (CH_2), 38.7 (CH_2), 38.9 (CH_2), 47.2 (CH_2), 47.5 (CH_2), 68.5, 68.7, 70.0, 70.0, 70.2, 70.3 (CH_2 PEG), 119.3 (CH_{Ar}), 129.5 (CH_{Ar}), 131.6 (C_{qt}), 142.6 (C_{qt}), 165.7 (C=O), 171.6 (C=O), 171.7 (C=O), 171.8 (C=O), 172.4 (C=O). **HR-ESI-MS** *m*/*z* calcd. for $[C_{60}H_{75}N_6O_8+2H]^{2+}$ 504.78960, found 504.78962. **RP-HPLC** (C18 analytical HTec Nucleodor Column, 254 nm): R_t = 8.82 min. Analytical RP-HPLC method: Nucleodur EC 250/4, 4 mm ID × 250 mm (C18 HTec, 5 µm) at a flow rate of 1 mL min^{-1} with a gradient of A (distilled H_2O containing 0.1% vol/vol TFA) and B (MeOH containing 0.1% vol/vol TFA): *t* = 0-2 min, 40% B; *t* = 2-9 min, 95% B; *t* = 11 min, 5% B.

PAUSE POINT Compound **1** can be stored in the dark at -80 °C for at least 3 months.

- **END OF BOX 1 -**

MATERIALS

REAGENTS

- Antibody or protein stock solution (typically 0.1 – 10 mg total protein mass; at a stock protein concentration ≥0.5 mM). CRITICAL: in our experiments with trastuzumab (formulated as Herceptin™, Genentech, Inc., South San Francisco, United States of America) and onartuzumab (formulated as MetMAb™, Genentech Inc.) we found that pre-purification of the antibody from the formulation buffer is unnecessary.[13,15,18,20] Commercially sourced stock solutions of MetMAb™ (typical protein concentration of 60 mg mL^{-1}) are formulated in 10 mmol L^{-1} histidine succinate, 106 mmol L^{-1} α,α-trehalose dihydrate, and 0.02% (vol/vol) polysorbate 20 at pH5.7.[24] Stock solutions of Herceptin™ (typical protein concentration of >15 mg mL^{-1}) were prepared by reconstitution of the Good Manufacturing Practise (GMP)-grade lyophilised powder in water. A standard vial of Herceptin™ contains trastuzumab (440 mg), L-histidine hydrochloride (9.9 mg), L-histidine (6.4 mg), α,α-trehalose dihydrate (400 mg, α-D-glucopyranosyl-α-D-glucopyranoside), and polysorbate 20 (1.8 mg). Stocks of Herceptin™ can be stored as lyophilised powders or as solutions at 4 ºC for >1 year. Stock solutions of MetMAb™ can be aliquoted and stored at –20 ºC for > 1 year.
- Photoactivatable aryl azide compounds such as: DFO-PEG_3-ArN_3 (**1**) ($C_{46}H_{77}N_{11}O_{14}$; molecular weight = 1,008.19 g mol^{-1}); DFO-PEG_3-$EtArN_3$ (**2**) ($C_{48}H_{81}N_{11}O_{14}$; molecular weight = 1,036.24 g mol^{-1}); DFO-ArN_3 (**3**) ($C_{32}H_{51}N_9O_9$; molecular weight = 705.81 g mol^{-1}). Structures of compounds **1**–**3** are presented in **Figure 2**. CRTICAL: Compounds **1**–**3** are prepared synthetically, in-house. The synthesis of compound **1** is described in Box 1. Small samples of the photoactivatable chelates are available upon request from the

corresponding author. Synthetic details and full characterisation data for compounds **1**–**3**, including ^{1}H- and ^{13}C{^{1}H}-NMR spectroscopy, high-resolution electrospray ionisation mass spectrometry (HRMS-ESI), high-performance liquid chromatography, electronic absorption spectroscopy, photochemical reactivity, and concordant data on the metallation complexes with natural $ZrCl_4$ or radioactive $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ have been reported elsewhere.[15,18,20] Photoactivatable derivatives of DFO can be stored in the dark at –80 ºC for at least 3 months. The chemical purity should be checked by HPLC prior to use.

- Distilled, deionised water (Milli-Q; >18.2 M MΩ·cm at 25 ºC) treated with Chelex® 100 sodium form (50-100 mesh; dry) resin (CAS no. 11139-85-8; Sigma-Aldrich, cat. no. C7901).
- Sodium carbonate: (Na_2CO_3·$10H_2O$; molecular weight = 286.14 g mol^{-1}; 99.999% trace metal basis; CAS no. 6132-02-1; Merck, cat. no. 577782; see REAGENT SETUP))
- Hydrochloric acid: (HCl; 37 wt%; 99.999% trace metal basis; molecular weight = 36.46 g mol^{-1}; CAS no. 7647-01-0; Merck, cat. no. 339253; see REAGENT SETUP)
- Phosphate buffered saline: (normal 1X PBS at pH7.4; prepared from a 10X stock solution by dilution with water; Merck, cat. no. P5493)
- Diethylenetriaminepentaacetic acid: (DTPA; $C_{14}H_{23}N_3O_{10}$; molecular weight = 393.35 g mol^{-1}; CAS no. 67-43-6; Merck, cat. no. D6518; see REAGENT SETUP)
- HPLC eluents (reverse-phase): Distilled, deionised water (Milli-Q; >18.2 M MΩ·cm at 25 ºC) and methanol (HPLC grade, >=99.9%; CAS no. 67-56-1, cat. no. 34860) containing 0.1% (vol/vol) trifluoroacetic acid (CF_3COOH; molecular weight = 114.02 g mol^{-1}; 99%; CAS no. 76-05-1; see REAGENT SETUP)

- ITLC eluents: Diethylenetriaminepentaacetic acid aqueous solution (50 mM; pH7.4): (DTPA; $C_{14}H_{23}N_3O_{10}$; molecular weight = 393.35 g mol^{-1}; CAS no. 67-43-6; Merck, cat. no. D6518; see REAGENT SETUP)
- $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ (^{89}Zr-oxalate) in ~1.0 M oxalic acid: (Radioactivity was purchased from a commercial vendor: PerkinElmer, Boston, United States of America but samples were manufactured and distributed by the BV Cyclotron VU, Amsterdam, The Netherlands). **! CAUTION** Zirconium-89 ($t_{1/2}$ = 78.41 h, $E_{max}(\beta^+)$ = 902 keV, $I(\beta^+)$ = 22.74%, $E\gamma$ = 909.2 keV [I = 99.0%], 511.0 keV [I = 45.5%]) emits positrons and high-energy gamma rays. All operations must be performed by qualified personnel in an approved facility and following safety guidelines set forth by the local authorities, and the Nuclear Regulatory Commission. Experimental manipulations should first be practised using non-radioactive samples and researchers should follow the ALARA (As Low As Reasonable Achievable) protocols to minimise exposure to ionising radiation. The use of all appropriate personal protective equipment including lab coats, shoes, gloves, plastic safety glasses and radiation dosimetry monitoring devices including whole body and finger ring detectors, as well as audible personal dose rate monitors and contamination monitors is mandatory when handling radioactive materials. **♦ CRITICAL** The ^{89}Zr-oxalate stock solution is highly acidic and must be neutralised before use (See REAGENT SETUP)

EQUIPMENT

- pH indicator strips: (Merck Millipore; variable ranges including pH 0–14 [cat. no. 109535]; pH 4 – 7 [cat. no. 109542]; pH 6.5 – 10 [cat. no. 109543])

- Calibrated automatic pipettes and tips: (volume ranges: 20 – 200 μL for reagent aliquots; 0.5 – 10 μL for analytical ITLC measurements)
- Calibrated pH meter: (SevenCompact Advanced pH Meter; Mettler Toledo, Greifensee, Switzerland, cat. no. S210-Std-Kit)
- Calibrated analytical balance: (XSR205DU, Mettler Toledo, Greifensee, Switzerland, cat. no. 30355396)
- Transparent glass sample vials: (2.0 mL screw vials 12×32 mm, Wicom Germany GmbH, Heppenheim, Germany, cat. no. WIC 41155-SIL)
- Microcentrifuge tubes: (1.5 mL; Eppendorf LoBind; Merck, cat. no. Z666505)
- Falcon tubes: (50 mL, self-standing, polypropylene; Corning New York, United States of America, cat. no. 430921)
- Magnetic stir bars: (PTFE ∅ 3×8 mm; Semadi AG, Ostermundigen, Switzerland, cat. no. 0244)
- Retort stand and clamp
- Magnetic stirring plate: (For example, IKA® RCT basic IKAMAG™ safety control; Merck, cat. no. Z645060; a stirring rate <1000 rpm to avoid aggregation of the protein)
- Silica-gel impregnated on glass fibre instant thin-layer chromatography strips: (ITLC; Agilent Technologies, cat. no. SGI0001)
- Geiger Counter: (Ranger EXP Geiger Counter with external pancake detector, Mineralab, New York, United States of America)
- Calibrated activimeter (dose calibrator): ISOMED 2010 (Nuklear-Medizintechnik, Dresden, Germany)
- Radio-ITLC detector: (SCAN-RAM, LabLogic Systems Ltd, Sheffield, United Kingdom).

- HPLC systems with electronic absorption and radioactive detection (γ or β^+) connected in series. In our lab, analytical HPLC experiments on small-molecules and complexes are performed by using Hitachi Chromaster Ultra Rs systems fitted with a reverse phase VP 250/4 Nucleodur C18 HTec (4 mm ID x 250 mm, 5µm) with electronic absorption measured at 220, 254 or 280 nm. Automated size-exclusion chromatography on protein samples is performed by using a Rigol HPLC system (Contrec AG, Dietikon, Switzerland) with electronic absorption measured at 280 nm. Both HPLC systems are connected to a radioactivity detector (FlowStar^2 LB 514, Berthold Technologies, Zug, Switzerland) equipped with a 20 µL PET cell (MX-20-6, Berthold Technologies).
- Size-exclusion chromatography gel filtration column: (ENrich SEC 70 or 650, 10 ± 2 µm, 10 mm ID x 300 mm; Bio-Rad Laboratories, cat. no. 780-1070 or 780-1650, respectively)
- Disposable PD-10 desalting columns for manual analytical and preparative size-exclusion chromatography: (Sephadex® G-25 resin, 85-260 µm, 14.5 mm ID x 50 mm, >30 kDa, GE Healthcare; Merck, cat. no. GE17-0851-01). CRITICAL: the loading/dead-volume of the PD-10 columns is precisely 2.50 mL which was discarded prior to aliquot collection.
- Calibrated automatic gamma counter for accurate quantification of radioactivity: Experiments used a HIDEX Automatic Gamma Counter (Hidex AMG, Turku, Finland)
- Light-emitting diodes with digital power control: Experiments used portable light-emitting diodes (LED; 365 nm or 395 nm) operated by a digital UV-LED controller (Opsytec Dr. Gröbel GmbH, Ettlingen, Germany). Spectroradiometric experiments confirmed that a setting of 100% corresponded to a power of approximately 263 mW at 365 nm and 355 mW at 395 nm. The LED (365 nm) had a maximum emission intensity at 364.5 nm (full-

width at half-maximum [FWHM] = 9.1 nm). The LED (395 nm) had a maximum emission intensity at 389.9 nm (FWHM = 9.1 nm). (see **Figure 4**)

REAGENT SETUP

Sodium carbonate solution (1.0 M in water): Add 14.31 g $Na_2CO_3 \cdot 10H_2O$ to a 50 mL Falcon tube and dissolve in water (50 mL). This solution can be stored at room temperature (23 ± 2 °C) for >1 year.

Hydrochloric acid solution (0.1 M in water): First prepare a 5.0 M solution of HCl by adding 20.53 mL HCl (37 wt %, d = 1.2 g mL^{-1} at 25 °C, [HCl] = 12.17 M) to a 50 mL Falcon tube containing 29.67 mL water. Prepare a 0.1 M HCl working stock by adding 1.0 mL 5.0 M HCl to a 50 mL Falcon tube containing 49.0 mL water. This solution can be stored at room temperature for >1 year. **! CAUTION** Acid dilution in water is highly exothermic. Allow the mixture to cool to ambient temperature before proceeding.

ITLC eluent (50 mM DTPA at pH7.4): Add 0.983 g DTPA to a 50 mL Falcon tube and add 30 mL water. Adjust the pH to 7.4 by adding aliquots of 1.0 M Na_2CO_3(aq.) solution and check the pH with a calibrated pH meter. Ensure that all of the white powder has dissolved before proceeding then make up the volume to a total of 50 mL by adding additional water. This solution can be stored at room temperature for >1 year.

HPLC eluent (reverse-phase): Solvent A = water with 0.1% v/v TFA. Solvent B = MeOH with 0.1% v/v TFA. These solvent mixtures can be stored at room temperature for >1 year.

DFO-PEG$_3$-ArN$_3$ solution (~0.2 mM, pH 8.0 – 8.5): Prepare a stock solution by dissolving 0.75 mg compound **1** (see Box 1) in 300 µL water. Check the pH by removing <1 µL aliquots and spotting on pH indictor strips. When necessary, adjust the pH first with small aliquots (typically <10 µL) of 1.0 M Na_2CO_3(aq.) and 0.1 M HCl(aq.) stock solutions. Dilute the ligand solution with water to give a clear colourless solution in a final volume of 350 µL (n(**1**) = 0.744 µmol, [**1**] = 2.13 mM). Compounds **1** and **2** are water soluble. However, to facilitate dissolution of the aggregated particulates that form during lyophilisation, a compound can first be dissolved in DMSO (≤10 µL; giving <3% v/v DMSO:water in the stock solution). Solutions of compounds **1** and **2** should be prepared fresh and can be stored at 4 °C for up to 1 week. The presence of <15% v/v DMSO in the reaction mixture does not interfere with the photochemical conjugation chemistry. ♦ **CRITICAL** DFO-ArN$_3$ (**3**) has very low solubility in water. The compound dissolves at pH>11 but slowly precipitates as a white amorphous powder when the pH of the mixture is adjusted (pH 8.0 – 8.5) with 0.1 M HCl. Compound **3** is soluble in DMSO and if the protein or antibody remains stable, a higher ratio of DMSO:water up to 30% v/v can be used. Solutions of DFO-ArN$_3$ (**3**) should be prepared fresh and used immediately.

Neutralised [^{89}Zr][Zr(C$_2$O$_4$)$_4$]$^{4-}$ stock solution: Add an aliquot of the ^{89}Zr-oxalate radioactivity from the commercial source (68.7 MBq, 70 µL in ~1.0 M aq. oxalic acid) to a glass vial containing water (200 µL). Neutralise and adjust the pH to slightly basic by the addition of aliquots of Na_2CO_3(aq.) (1.0 M stock solution; final pH ~8.3 – 8.5). **! CAUTION** Acid neutralisation with Na_2CO_3(aq.) releases CO_2(g). Care should be taken to ensure that no radioactivity escapes the vial.

Test the pH by removing an aliquot of the solution (<1 μL) and spotting on pH indicator strips (range 6.5 – 10.0). If the solution becomes too basic, the pH can be adjusted by using aliquots of 0.1 M HCl(aq.). ♦ **CRITICAL** For optimum radiolabelling results, the neutralised solutions of $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ should be used within 24 h after preparation ensuring that the pH remains at the correct value before use.

EQUIPMENT SETUP

CRITICAL All instruments for measuring radioactivity should be calibrated and maintained in accordance with previously reported routine quality control procedures.[25]

Radio-ITLC detector: Turn on the instrument and allow the detector to warm up for 15 min before use. Run a background scan using the same parameters as employed for the analytical samples to ensure that no radioactive contaminants are present on the scanner bed.

HPLC method for analytical measurements: Turn on the electronic absorption and radioactivity detectors, and equilibrate the HPLC gel filtration column for 30 min before use. Run a blank sample injecting ~20 μL of water (reverse-phase) or PBS (SEC) to confirm that no impurities are present on the column or in the injection loop. For reverse-phase HPLC: use a gradient elution profile of 40% B (0 min) to 95% B (11 min) at a flow rate of 1.0 mL min^{-1}. For SEC gel filtration: use an isocratic elution profile with sterile PBS (1X, pH7.4) at a flow rate of 1.0 mL min^{-1}.

Gamma counter: Use an energy window of 480 – 558 keV for ^{89}Zr (511 keV emission) and a counting time of 30 seconds. Apply appropriate background and decay corrections throughout.

Activimeter: Change the dose calibrator settings to measure ^{89}Zr radioactivity. Measure a background sample to ensure that the dose calibrator is not contaminated before use.

Geiger counter: Check the battery power and voltage.

LEDs: Turn on the digital control box and allow the electronics to stabilise for 15 min at ambient temperature before use.

PD-10 preconditioning: Before use, cut the plastic end (tip) to open 3 fresh PD-10 desalting columns (per radiolabelling experiment). Remove the cap and discard the storage solution. Rinse each column with 4×5 mL sterile 1X PBS. After preconditioning, add 1 mL PBS to the top of the column, cap both ends, and store until required (max. 24 h before use).

PROCEDURE

Photoradiolabelling of mAbs with ^{89}Zr and DFO-PEG$_3$-ArN$_3$ • TIMING 40 min

! CAUTION Zirconium-89 is radioactive and emits positrons and high-energy gamma rays. This material should only be handled by trained personnel in approved radioactive facilities. Further details on the radioactivity are listed in the Materials section.

♦ **CRITICAL** The temperature of all photochemical conjugation reactions is 23 ± 2 °C (ambient conditions). Although it is conceivable that slightly higher or lower temperatures can be used, reactions involving proteins should not exceed 37 °C to avoid thermal denaturation.

1. Based on the known concentrations, calculate the volumes required of the stock solutions of protein, [^{89}Zr][Zr(C$_2$O$_4$)$_4$]$^{4-}$, and DFO-PEG$_3$-ArN$_3$ (**1**). For example:
    - **mAb aliquot:** For a full-sized IgG$_1$ antibody with a nominal molecular weight of 150,000 Da, and a stock protein molality of 50 mg mL^{-1} ([protein] = 0.33 mM), an aliquot of ≥45 µL would give a final protein concentration of ≥0.1 mM (equivalent to a total *n*(protein) ≥15.0 nmol or 2.25 mg of protein).
    - **DFO-PEG$_3$-ArN$_3$ (1) aliquot:** For a stock concentration of 2.13 mM and desired chelate-to-antibody ratio = 1.5, the [**1**] required (given by [protein] × initial chelate-to-protein ratio) = 0.1 mM × 1.5 = 0.15 mM. Volume required = 10.6 µL (equivalent to *n*(**1**) = 22.5 nmol).
    - **[^{89}Zr][Zr(C$_2$O$_4$)$_4$]$^{4-}$ aliquot:** The molar activity of the neutralised ^{89}Zr-stock solution can be determined experimentally by titration with a known amount of DFO. Alternatively, some manufacturers provide this information in the materials specification and data sheets. A conservative value for the molar activity of [^{89}Zr][Zr(C$_2$O$_4$)$_4$]$^{4-}$(aq.) is A_m = 37.0 ± 0.12 MBq nmol^{-1} with activity concentrations in the range >2 GBq mL^{-1}.[15] At this value of A_m, DFO-PEG$_3$-ArN$_3$ can be radiolabelled with ~830 MBq of ^{89}Zr (assuming 1:1 stoichiometry of the

metal-to-ligand complex). For preclinical work, an activity of ≤74 MBq (≤2 mCi) in 20 µL is typically used. This implies that more than a 10-fold excess of chelate is present in the initial reaction mixture ensuring quantitative and rapid ^{89}Zr-radiolabelling of compound **1** in <1 min under ambient conditions.[26]

- **Water balance:** The amount of water required is equal to the final reaction volume minus the sum of the reagent aliquots. Here, $V(H_2O)$ = (150 µL – 75.6 µL) = 74.4 µL.

♦ **CRITICAL STEP** The reaction mixture should contain a minimum protein concentration of 0.1 mM. Conjugation yields decreased when protein concentrations below 0.1 mM were used.[18]

♦ **CRITICAL STEP** The final reaction volume should be kept to a minimum (typically, V_{total} = 150 µL). The initial chelate-to-protein molar ratio can be varied freely but should ideally be in the range 1:1 or 2:1.

♦ **CRITICAL STEP** For radioactive work, it is important to ensure that enough chelate is present to complex the radionuclide.

2. Pipette the required amount of water into the transparent glass vial equipped with a magnetic stir bar.
3. Pipette the required volume of the photoactivatable reagent DFO-PEG$_3$-ArN$_3$ (**1**) into the glass vial and place the vessel on a magnetic stirring plate with rotation speed set at <1000 rpm.
4. Inside a lead shielded hood, pipette the desired amount of radioactivity (typically ≤74 MBq) into the reaction. Measure the activity in the reaction vial by using the calibrated activimeter and record the time. This measurement is the initial activity at the start of

reaction ($A_{initial}$ / MBq; t = 0.0 min). With efficient mixing, the complexation of $^{89}Zr^{4+}$ ions by the DFO-PEG_3-ArN_3 (**1**) chelate is generally complete in 1 – 2 minutes.

! CAUTION All materials (including pipette tips, pH indicator paper and ITLC strips) that are used to handle, transfer or analyse radioactivity should be discarded after use in a labelled, plastic container, and stored behind a lead shield.

5. Using a pipette, take an aliquot (<1 μL) of the reaction mixture from Step 4 and spot onto an ITLC strip (10 mm × 150 mm) at ~30 mm from the bottom of the strip. The position of the spot is defined as R_f = 0.0. The position of the solvent front is defined as R_f = 1.0 and is located at the top of the ITLC strip. Transfer the spotted ITLC strip into a TLC chamber containing the ITLC eluent. Develop the plate for ~2 – 3 minutes until the solvent front reaches the top of the ITLC strip. Remove the ITLC strip, seal in transparent adhesive tape (cellotape) to avoid radioactive contaminations, and record the chromatogram by using the radio-ITLC plate reader (see Anticipated Results Figure 6A for representative radio-ITLC data). **? TROUBLESHOOTING**

♦ CRITICAL STEP Ensure that the level of the ITLC eluent in the TLC chamber does not exceed the R_f = 0.0 threshold on the strip.

♦ CRITICAL STEP Under these ITLC conditions, 'free' $^{89}Zr^{4+}$ ions are rapidly coordinated by DTPA to give the $[^{89}Zr][Zr(DTPA)]^-$ complex which migrates to the solvent front (R_f = 0.0). The desired species, $[^{89}Zr][Zr(DFO\text{-}PEG_3\text{-}ArN_3)]^+$ (also called ^{89}Zr-**1**$^+$) is retained at the baseline (R_f = 0.0). The percentage of radiochemical conversion yield (RCC / %) to give ^{89}Zr-**1**$^+$ can be estimated by integration of baseline activity (recorded in counts or counter per minute [CPM] in the range R_f

= 0.0 – 0.1), and the total CPM recorded over the entire strip (R_f = 0.0 – 1.0) from Equation 1. Background subtraction can also be performed where necessary.

$$\text{RCC}/\% = \frac{\sum_{R_f=0.0}^{R_f=0.1} \text{CPM}(^{89}\text{Zr}-\mathbf{1}^+)}{\sum_{R_f=0.0}^{R_f=1.0} \text{CPM}(\text{total})} \times 100\% \qquad \text{Equation 1}$$

6. Using a pipette, take another aliquot (<5 μL) of the reaction mixture from Step 4, transfer to a HPLC vial and (if necessary) dilute with 20 μL water. Analyse the formation of ^{89}Zr-**1**$^+$ by reverse-phase HPLC. Co-elution of the radioactive ^{89}Zr-**1**$^+$ species with an authenticated sample of non-radioactive ^{nat}Zr-**1**$^+$ can be used to confirm the identity of the radiolabelled compound. The procedure can be continued whilst the HPLC is acquiring data. **? TROUBLESHOOTING**
7. After confirming by radio-ITLC that ^{89}Zr-**1**$^+$ is formed *in situ*, pipette the required volume of protein stock solution into the reaction vial.
8. Check the pH by removing an aliquot (<1 μL) and spotting a pH indictor strip (acceptable range pH 8.0 – 8.5). **? TROUBLESHOOTING**
9. Place the LED (365 nm or 395 nm) in position and secure with a retort stand and clamp. Irradiate the sample directly from above (see **Figure 3**). The digital controller can be used to define the irradiation time and tune the power (typical settings: 100% power for 15 min).

♦ **CRITICAL STEP** The experimental geometry has a major impact on the flux of photons passing through the reaction mixture. Direct irradiation (with a correctly focused LED beam) of the open sample avoids potential issues with scattering, reflection and absorbance of the light by the glass. Specialised apparatus such as a mirrored photochemical light box can be used but the basic principle is that the focal point of the LED source should be located in the centre of the reaction mixture to ensure efficient and reproducible activation of the ArN_3 group. If the light is

transmitted to the sample through the reaction vessel, the use of a quartz cuvette will minimise absorption and scattering. Standard actinometry measurements can be used to determine the power delivered into the sample.[27]

10. After irradiation, remove the LED source. Using a pipette, take an aliquot (<1 μL) of the crude product from Step 9 and spot onto an ITLC strip. Develop and measure the ITLC strip as described in step 5. Radiolabelled protein is retained on the ITLC strip at $R_f = 0.0$ (see Anticipated Results Figure 6A for representative radio-ITLC data). This analysis will confirm if the photochemical reaction has liberated any free $^{89}Zr^{4+}$ ions due to decomposition of ^{89}Zr-**1**$^{+}$.
11. Using a pipette, take two further aliquots (2×5 μL) of the crude mixture from Step 9. Transfer one aliquot into a HPLC vial and analyse the crude product by SEC-HPLC and (if necessary) dilute with water (20 – 50 μL) (see Anticipated Results Figure 6C for representative SEC-HPLC data).
12. Transfer the second aliquot into an Eppendorf tube, dilute with 20 μL water, and retain for later analysis by manual analytical PD-10 size-exclusion chromatograph (see step 24).
13. Measure the remaining activity in the reaction vial from Step 9 by using the activimeter and record the time (A_{crude} / MBq; crude activity).
14. Remove the caps from a preconditioned PD-10 column, discard the PBS, and fix the column inside a lead shielded hood by using the retort stand and clamp. Place a labelled collection vial underneath the column (waste).
15. From the remaining crude reaction mixture from Step 13, use a pipette to transfer an aliquot (typically, V_{loaded} = 120 μL) to the top of the PD-10 column and allow the liquid to flow

under gravity. Check the pipette tip by using the activimeter or Geiger Counter to confirm that the activity was transferred correctly. With a fresh pipette tip, add successive fractions of 1X PBS (1×380 µL, then 2×1,000 µL) to obtain a total loading volume of 2.5 mL. Collect the eluate in the waste vial, measure the activity by using the activimeter and record the time (waste activity).

16. Measure the remaining activity in the crude reaction vial and record the time (A_{residual} / MBq; residual activity).
17. Place a sterile, labelled vial under the PD-10 column to collect the purified product. Elute the column with 1.6 mL (2 × 800 µL) (acceptable range: 1.5 – 2.0 mL) 1X PBS and collect the product fraction. The eluate contains the high-molecular weight fraction of purified protein but may also contain other species that have a nominal molecular weight >30,000 Da. Continue to elute the PD-10 column with successive fractions of 1X PBS (2 × 1 mL) three times (fractions 2, 3, and 4).
18. Measure the activity in the protein fraction and record the time (A_{purified} / MBq; purified product activity for fraction 1 at t = end of synthesis [EOS]).
19. Measure the activity eluted fractions 2, 3, and 4, as well as the residual activity on the PD-10 column.
20. Calculate the decay-corrected radiochemical yield (RCY / %) of the isolated, radiolabelled protein in accordance with Equation 2. The ratio V_{total} / V_{loaded} corrects for the fact that only a 120 µL fraction of the reaction mixture was purified in step 15.

♦ **CRITICAL STEP** All measured activities (measured in consistent units of either MBq or mCi) must be decay-corrected to the same time point before performing the calculation (typically, t = EOS).

$$\text{RCY} / \% = \left(A_{\text{product}} \cdot \frac{V_{\text{total}}}{V_{\text{loaded}}}\right) / A_{\text{initial}} \qquad \text{Equation 2}$$

? TROUBLESHOOTING

21. Using a pipette, take three separate aliquots of the purified product from Step 18. Spot the first aliquot (<5 µL) spot onto an ITLC strip. Develop and measure the ITLC strip as described in step 5.
22. Transfer the second aliquot (20 – 50 µL) into a HPLC vial and analyse the purified product by SEC-HPLC. **? TROUBLESHOOTING**
23. Transfer the third aliquot (20 – 50 µL) into an Eppendorf tube and retain for analysis by manual analytical PD-10 size-exclusion chromatograph in Steps 24-28.

□ PAUSE POINT The crude and purified aliquots that were retained for manual PD-10 SEC analysis should ideally be analysed immediately but can be stored at 4 ºC for up 24 h.

Manual PD-10 size-exclusion analysis • TIMING 25 min per sample

24. Remove the caps from a preconditioned PD-10 column, discard the PBS, and fix the column inside a lead shielded hood by using the retort stand and clamp. Place a labelled collection vial underneath the column (waste).
25. Using a pipette, transfer 20 µL of the retained crude reaction mixture from the Eppendorf tube (Step 12) on to the PD-10 column and allow the liquid to flow under gravity. With a fresh pipette tip, add successive fractions of 1X PBS (1 × 480 µL, then 2 × 1,000 µL) to obtain a total loading volume of 2.5 mL. Collect the eluate in the waste vial, measure the activity by using the activimeter and record the time (waste activity).

26. Elute the PD-10 column with (40 × 200 µL) fractions of 1X PBS collecting and capping each fraction in a separate gamma count vial. **? TROUBLESHOOTING**

♦ **CRITICAL STEP** Accuracy in collecting the 200 µL eluate fractions is crucial. Ensure that residual drops are collected from the end of the PD-10 column before changing the collection vial to prevent large errors in the measured PD-10 elution profiles.

27. Repeat steps 24 – 26 using a fresh preconditioned PD-10 column for the aliquot of the purified product from Step 23.
28. Measure the samples and appropriate blank tubes using a gamma counter. Data from the analytical PD-10 measurements (Steps 24-27) or the radioactive HPLC-SEC traces (Steps 11 and 22) can be used to estimate the radiochemical purity of both the crude and purified samples.

♦ **CRITICAL STEP** Each set of analytical PD-10 samples should be measured alongside a set of 4 blank tubes to measure the background. The gamma count data for each sample should be decay-corrected to the same time point before plotting the elution traces and performing other calculations from these data.

● **TIMING**

CRITICAL: All stock solutions and equipment should be prepared in advance and are not included in the timing.

Box 1, Synthesis and Characterisation of DFO-PEG_3-ArN_3 (**1**): 6 days

Steps 1 – 23, Photoradiolabelling of mAbs with ^{89}Zr and DFO-PEG_3-ArN_3: 40 min

Steps 24 – 26, Manual PD-10 size-exclusion analysis: 25 min per sample

Step 28, Gamma counter analysis: timing depends on the counting time and time required for the robot to manoeuvre each tube or rack into position. A 30 second count of each tube would typically require an additional 30 seconds.

? TROUBLESHOOTING

Troubleshooting advice is presented in **Table 1**.

Table 1. Troubleshooting table.

Step	Problem	Possible reasons	Possible solution
5	Radio-ITLC analysis indicates incomplete formation of ^{89}Zr-**1**$^{+}$	• Insufficient reaction time • Incorrect pH • Low molar activity of the $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ or the presence of chemical impurities such as Fe^{3+} or Ga^{3+} ions.	• Leave the reaction for a further 5 min and repeat the ITLC analysis • Check and adjust the pH to 8.0 – 8.5. NOTE: $^{89}Zr^{4+}$ metallation of DFO derivatives occurs efficiently at pH 5 – 10, however, one-pot photoradiochemistry requires slightly basic conditions. • Check the molar activity of the neutralised $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ • Discard solvents and prepare fresh buffer solutions using metal free reagents and Chelex® 100 resin treated distilled, deionised water to remove metal ion contaminants
6	Reverse-phase radio-HPLC analysis indicates incomplete formation of ^{89}Zr-**1**$^{+}$ or the presence of free $^{89}Zr^{4+}$ ions	• Insufficient reaction time • Low molar activity of the $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ or the presence of chemical impurities such as Fe^{3+} or Ga^{3+} ions.	• See Troubleshooting for Step 5 • It is important to note that free $^{89}Zr^{4+}$ ions will bind to protein non-specifically, however, the reaction conditions typically involve an excess of DFO chelate which is usually sufficient to complex all available $^{89}Zr^{4+}$ ions and remove any activity that is bound non-specifically to the protein.

8	Incorrect pH	• Impurities in the water or inaccurate pH of the stock solutions.	• Check and adjust the pH (8.0 – 8.5) of the stock solutions of DFO-PEG_3-ArN_3 and $[^{89}Zr][Zr(C_2O_4)_4]^{4-}$ by using aliquots of 1 M Na_2CO_3(aq.) or 0.1 M HCl(aq.) and spotting on pH indicator strips.
20	Low RCYs	• Incorrect geometry of the photochemical apparatus • Incomplete photochemical activation of the ArN_3 compound.	• Check that the light is correctly focused on the reaction solution. • Perform a control experiment by omitting the protein component and replacing the volume with water. After irradiation of the ^{89}Zr-**1**$^+$ solution, remove an aliquot and analyse by reverse-phase HPLC. The peak associated with ^{89}Zr-**1**$^+$ should be absent and the photolysis products are more polar and typically elute at shorter retention times. • See ANTICIPATED RESULTS for further comments on the observed RCYs from photoradiolabelling of proteins.
22	SEC-HPLC analysis shows <90% radiochemical purity	• PD-10 desalting columns are suboptimal for separating some small-molecule metal-ion complexes from the protein. Experience has shown that standard PD-10 columns are only efficient when the radiochemical purity of the crude mixture is >75%. For mixtures with higher fractions of radiolabelled small-molecule (<30,000 Da) components, separation efficiency decreased.	• Consider using alternative gel filtration media such as polyacrylamide gels (Bio-Rad, P-4 gel, cat. no. 1504124; P-6 gel, cat. no. 1504130) • Prepare a longer gel filtration column that will increase the separation between high- and low- molecular weight species. • Use centrifugal size-exclusion spin-filtration methods to concentrate and purify the product further.

			• Using a concentrated sample of the product, repeat the PD-10 purification (Steps 14 – 18)
26	• Inaccurate elution profiles from analytical PD-10 measurements show large errors or the activity peak for the protein fraction is not found in the 1.0 – 1.8 mL window • PD-10 stops flowing or runs very slowly	• Inaccurate loading of sample and water (Step 25) onto the PD-10 column or inaccurate collection of the 200 µL fractions (e.g. loss of a drop during step 26) • Precipitates (from the protein or other components of the reaction mixture block the pores in the gel	• Repeat the PD-10 analysis with a fresh column. • See CRITICAL STEP note in Step 26

ANTICIPATED RESULTS

As a representative example, details on the photoradiosynthesis of [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab are given in **Figure 5**. Results obtained from the radio-ITLC, analytical PD-10 elution profiles and SEC-HPLC experiments for the synthesis of [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab are presented in **Figure 6**. Radio-ITLC data indicate the efficient radiolabelling of compound **1** with [^{89}Zr][Zr(C_2O_4)$_4$]$^{4-}$ and the facile separation of ^{89}Zr-**1**$^+$ (green trace) from the control [^{89}Zr][Zr(DTPA)]$^-$ (black trace). Under the radio-ITLC conditions employed, ^{89}Zr-radiolabelled proteins are retained at the baseline (R_f = 0.0). Analytical PD-10 elution profiles from the crude (red) and purified (blue) protein mixtures can be used to estimate the radiochemical purity. The marker set at 1.6 mL indicates the cut-off used to isolate the radiolabelled [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab during preparative PD-10 chromatography (Step 17). Size-exclusion HPLC analysis is a more reliable way to estimate the radiochemical purity of the final product. Co-elution of the radiolabelled protein with the UV trace (purple) from MetMAb^{TM} confirms the identity of the product as an ^{89}Zr-mAb. In addition, SEC-HPLC can reveal the presence of aggregated protein (*) or small-molecule by-products from the photolysis of ^{89}Zr-**1**$^+$ ($^{\Delta}$). Experiments found that the molar activity of ^{89}Zr-mAbs produced by photoradiosynthesis can be >13 MBq nmol^{-1}.[15,18] We note that this number is mostly limited by the fact that our facility is only equipped to handle small amounts of ^{89}Zr radioactivity in manual syntheses. Higher molar activities are likely to be achieved by facilities that can perform radiolabelling reactions with higher initial amounts of radioactivity. Cellular binding assays also confirmed that photoradiolabelled mAbs remain immunoreactive.[15,16,18,20] PET imaging studies in athymic nude mice bearing subcutaneous tumours derived from the MKN-45 gastric carcinoma cell line revealed

that [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab is a viable radiotracer for measuring the expression of the human hepatocyte growth-factor receptor (c-MET) (**Figure 7**).[20]

Optimisation studies revealed that, alongside standardisation of the experimental geometry, changes in the protein concentration have a major impact on the isolated RCYs (**Table 2**). Initial experiments with ^{89}Zr-**3**$^{+}$ encountered problems due to the limited solubility of the DFO-ArN$_3$ (**3**) compound in aqueous conditions.[15] Synthesis of the water-soluble chelates **1** and **2**, improved the reliability and RCYs for ^{89}Zr-radiolabelling of several proteins.[20] Decay-corrected RCYs of the isolated ^{89}Zr-photoradiolabelled proteins still show dependence on the nature of the protein and on the composition of the formulation buffer. In our experiments, photoactivatable [^{89}Zr]ZrDFO complexes derived from compound **1**, **2**, and **3**, have (so far) resulted in the highest RCYs of conjugated protein. The use of other photoactivatable metal binding chelates (including well-known polyazamacrocycles, HBED-CC-ArN$_3$, and DTPA-ArN$_3$) with ^{68}Ga^{3+} or ^{111}In^{3+} ions gave radiolabelled protein but with lower RCYs.[13,14,16,17]

For photochemical conjugation reactions using ArN$_3$, experimental evidence indicated that radiochemical conversions to the labelled protein decrease below pH 7 and above pH 9.1.[13] At neutral or acidic pH, free primary amines on the protein are likely to be fully protonated (pK_a [lysine ε-NH$_2$] = 10.5), and are not available to attack the ketenimine electrophile that is produced after light-induced activation of the ArN$_3$ group. At pH >9, quenching by hydroxide can become rate limiting. Further experimental and computation studies are underway to improve RCYs, to develop photoactivatable fluorophores[21] and cytotoxic drugs, and to explore the chemical scope and mechanisms of light-induced chemistry for making protein conjugates.

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AUTHOR CONTRIBUTIONS

JPH designed and supervised the project, provided funding, and wrote the original manuscript. AG, DFE and MP synthesised and characterised the photoactivatable DFO compounds. All authors performed the radiochemistry and analysis. JPH is the study director and resource manager on the approved animal experimentation license. JPH performed all PET imaging and biodistribution experiments and was assisted by AG and MP. All authors reviewed and approved the manuscript. AG and DFE contributed equally to this work. JPH is the senior/corresponding author.

COMPETING INTERESTS STATEMENT

The authors declare that there are no competing financial interests.

DATA AVAILABILITY

Source data for the analytical chromatography and PET images are available from the corresponding author.

RELATED LINKS

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FIGURES AND FIGURE LEGENDS

Figure 1. The general concept of light-induced activation and protein functionalisation.

Figure 2. Chemical structures of three photoactivatable DFO derivatives.

The structures of compounds 1 – 3 are shown. Note, the water-soluble version, DFO-PEG_3-ArN_3 (**1**) incorporates a discrete *tris*-polyethylene glycol (PEG_3) spacer between the metal ion binding moiety and the ArN_3 group. (Adapted from Guillou *et al.*, Chem. Eur. J., **26**, 7185-7189 (2020)).[20]

Figure 3. Photochemical apparatus and experimental setup.

A simple experimental setup for direct irradiation of a sample in an open vial is shown. More sophisticated experimental apparatus (such as light boxes) can be used for performing photochemical reactions. (Adapted from Patra *et al.*, Angew. Chemie Int. Ed., **58**, 1928–1933 (2019). DOI: 10.1002/anie.201813287).[13]

Figure 4. Emission spectra and beam shape.

Emission spectra of two LED sources with peak emissions at 365 nm (blue) and 395 nm (black). (Inset) Photograph of the emission geometry and focal point located ~1 cm from the lens. (Adapted from Patra *et al.*, Angew. Chemie Int. Ed., **58**, 1928–1933 (2019). DOI: 10.1002/anie.201813287).[13]

Figure 5. Simultaneous photoradiosynthesis of monovalent (one-armed) [^{89}Zr]ZrDFO-PEG_3-azepin-onartuzumab.

The reaction starts from fully formulated MetMAb^{TM}, DFO-PEG$_3$-ArN$_3$ (**1**), and [^{89}Zr(C$_2$O$_4$)$_4$]$^{4-}$ (^{89}Zr-oxalate).

Figure 6. Analytical data on the synthesis and characterisation of [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab.

Representative analytical data from the photoradiosynthesis of [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab starting from DFO-PEG$_3$-ArN$_3$ (**1**), [^{89}Zr][Zr(C$_2$O$_4$)$_4$]$^{4-}$, and formulated MetMAb^{TM} are shown. **a** Radio-ITLC chromatograms, **b** analytical PD-10 elution profiles, and **c** SEC-HPLC chromatograms of [^{89}Zr][Zr(DTPA)]$^{-}$ (black), ^{89}Zr-**1**$^{+}$ (green), [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab crude (red) and purified products (blue) and the UV trace of MetMAb^{TM} (purple) showing co-elution with the radiolabelled protein peak. * peak associated with a minor protein aggregate fraction; $^{\Delta}$ broad peak associated with radiolabelled small-molecule biproducts from the photolysis of ^{89}Zr-**1**$^{+}$. (Adapted from Guillou *et al.*, Chem. Eur. J., **26**, 7185-7189 (2020)).[20]

Figure 7. Representative PET images using [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab.

Coronal and axial PET images taken through the centre of the tumours showing the spatial distribution of [^{89}Zr]ZrDFO-PEG$_3$-azepin-onartuzumab over time after intravenous administration in mice bearing MKN-45 tumours. T = tumour, H = heart, L = liver, Sp = spleen. (Adapted from Guillou *et al.*, Chem. Eur. J., **26**, 7185-7189 (2020) – this reference contains full experimental details for the PET image acquisition and data analysis).[20] PET imaging was conducted in accordance with an animal experimentation protocol approved by the Zurich Canton Veterinary Office, Switzerland.

Table 2. Variations in radiochemical yields for photoradiolabelling of three different proteins with ^{89}Zr-**1**$^{+}$, ^{89}Zr-**2**$^{+}$ or ^{89}Zr-**3**$^{+}$.[15,18,20]

Compound	**Protein concentration [μM]** [a]	**Initial chelate-to-protein ratio**	**Irradiation time and wavelength [λ / nm]**	**Decay-corrected radiochemical yield (RCY) / % ± 1 standard deviation for *n* replicates**
^{89}Zr-**1**$^{+}$	HSA[b], 175 μM	1.2	15 min, 395 nm	72.9±1.9% (*n* = 3)
^{89}Zr-**1**$^{+}$	HSA, 175 μM	1.2	15 min, 365 nm	70.7±2.3% (*n* = 3)
^{89}Zr-**1**$^{+}$	MetMAb^{TM}, 141 μM	1.5	15 min, 395 nm	64.5±6.7% (*n* = 3)
^{89}Zr-**1**$^{+}$	Herceptin^{TM}, 77 μM	2.8	15 min, 395 nm	58.3±3.4% (*n* = 3)
^{89}Zr-**2**$^{+}$	HSA, 175 μM	1.2	15 min, 395 nm	59.6±3.6% (*n* = 3)
^{89}Zr-**3**$^{+}$	Trastuzumab, 51 μM	28.9	10 min, 365 nm	67 – 88% (*n* = 2)
^{89}Zr-**3**$^{+}$	MetMAb^{TM}, 38 μM 38 μM 38 μM	 4.6 2.3 0.8	10 min, 365 nm	 41.2 ± 10.6% (*n* = 3) 33.7 ± 5.8% (*n* = 3) 34.9 ± 10.4% (*n* = 3)

[a] Herceptin^{TM} and MetMAb^{TM} indicate that the formulated protein stocks were used directly without pre-purification of the protein. Trastuzumab indicates that the purified protein sample was used in the photoradiolabelling reactions.

[b] HSA = human serum albumin